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4. Title of the invention

BACULOVIRUS EXPRESSION SYSTEM

5. Name of your agent *(if you have one)*

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BACULOVIRUS EXPRESSION SYSTEM

The invention relates to a method for cloning a gene, to replication-deficient baculovirus vectors and rescue vectors for use in the method, to cells comprising such vectors and to kits comprising such vectors.

Baculoviruses have been isolated from a number of invertebrates. Most examples have been found in insect species, but there are some reports of baculoviruses which are pathogenic for crustacea. Baculovirus infections have been described in over 600 species of insects including Lepidoptera, Hymenoptera, Diptera and Coleoptera. Baculoviruses and their use as expression vectors are discussed in the book by King, L.A. and Possee, R.D. "The Baculovirus Expression System - A Laboratory Guide", Chapman & Hall, 1992.

Baculoviruses have a large, double stranded covalently-closed circular DNA genome of between 88 and 200 kbp. This associated with a highly basic (arginine-rich) protein of 6.5 kDa, within a rod-shaped nucleocapsid which contains a 39 kDa capsid protein. Other structural components almost certainly remain to be identified. The size of the virus genome determines the length of the nucleocapsid, which may be 200-400 nm.

The nucleocapsids are further packaged within a lipoprotein envelope to form a virus particle or virion. These structures may be occluded within a crystalline matrix or polyhedron consisting largely of a single protein (polyhedrin) of about 30 kDa, and form in the nucleus of infected cells. Polyhedra are large structures ranging in size from 1-15 μM in diameter with an outer polysaccharide envelope which appears to confer additional strength and protection.

Baculoviruses are usually named after the host from which they are isolated. For example, the baculovirus isolated from alfalfa looper was designated *Autographa californica* (Ac) MNPV. However, baculoviruses which are almost identical to Ac MNPV have been found in *Trichoplusia ni, Galleria mellonella and Rachiplusia ou.*

The Ac MNPV has been extensively studied at the molecular level largely because of its efficient replication in cell culture. In consequence, it was a logical example to be exploited as an expression vector. It should be mentioned however, that other baculovirus expression vectors are known, for example from *Bombyx mori* (silkworm) (Bm) NPV. This latter system, while particularly useful for producing recombinant proteins in silkworm larvae, which are easily reared and handled, has not achieved such widespread popularity as the Ac MNPV system.

Baculoviruses have been used as expression vectors. Genes from other species (e.g. humans, other vertebrates plants and bacteria) are inserted into the baculovirus genome (DNA) under the control of a very strong promoter (e.g. polyhedrin) to make a recombinant virus. This promoter drives expression of the foreign gene to make messenger RNA, which in turn makes protein in the recombinant virus-infected cell. The advantage of using this system is that the level of protein generated in the virus-infected cells may be several-fold higher than that achieved in the normal environment in which the protein is made. The function of the recombinant protein may subsequently be studied within the baculovirus-infected cell or after isolation of the product. The baculovirus expression system is widely used in industry and other research laboratories world-wide.

One of the limitations of the baculovirus expression system is that the process of inserting foreign genes into the virus genome is time consuming and labour intensive. Currently, two major methods are used. In the first, the baculovirus genome is maintained in *E. coli* and foreign genes are inserted via a process of transposon mutagenesis after introducing another plasmid (the transfer vector) into the bacterial cell. After recovering virus DNA from amplified bacterial cells, it is used to infect insect cells to produce a recombinant virus. In the second method, linear baculovirus DNA produced by digestion with a restriction endonuclease is mixed with the transfer vector and used to infect insect cells. The transfer vector serves to recircularise the virus genome and concomitantly insert the foreign gene. Unfortunately, the recombinant virus recovered from the infected cells is usually contaminated with a low

level of parental virus. This has to be removed by performing plaque purification in insect cells to derive clonal stocks of the recombinant virus. A single operator, using either method, cannot make more than 25 recombinant viruses per week.

Neither of the methods described above is readily amenable to the high throughput generation of recombinant viruses; both require too many manipulations. Such a method is now in demand because of the rapid deposition of sequence data from the human genome project (and other species) in the databases world-wide. To interpret such vast stores of information, it will be necessary to express thousands of genes in alternative hosts (e.g. insect cells) to derive sufficient material to determine protein function.

A fundamental and interesting question in baculovirus molecular biology concerns the regulation of virus gene expression. Most studies addressing this issue have utilised AcMNPV (Friesen, 1997; Lu and Miller, 1997). Three major phases of virus gene expression can be distinguished, according to the type of RNA polymerase utilised. Prior to virus DNA replication, early genes are transcribed by a host RNA polymerase II via promoters that resemble cellular regulatory elements (reviewed by Friesen, 1997). Late and very late genes are transcribed after the onset of DNA replication by a virus-encoded (α-amanitin-resistant RNA polymerase (Beniya *et al.*, 1996; Yang *et al.*, 1991; Huh and Weaver, 1990; Fuchs *et al.*, 1983; Grula *et al.*, 1981 and reviewed by Lu and Miller, 1997).

Recently, an RNA polymerase complex was identified and purified to homogeneity. It was shown to contain four equimolar sub-units encoded by *lef-4, p47, lef-8* and *lef-9* respectively (Guarino *et al.*, 1998). This enzyme transcribes genes containing the late/very late promoter/initiator sequence DTAAG (Blissard and Rohrmann, 1990; Lu and Miller, 1997). Preliminary experiments indicate that the core enzyme seems to be able to bind DNA, melt the duplex and accurately initiate RNA synthesis (Guarino *et al.*, 1998; Mans and Mörsdorf, 1998; Funk *et al.*, 1998). It is unclear, however, if the components of this enzyme are the sole determinants of late baculovirus gene

expression. Other factors are probably involved in the fine regulation of late gene expression and in the "burst" of the expression of very late genes.

Using transient-expression assays and temperature-sensitive mutants, a set of 20 virus-encoded factors were associated directly or indirectly in late and very late gene expression (Rapp *et al.*, 1998; Fan *et al.*, 1998; Todd *et al.*, 1995; 1996; Fan *et al.*, 1996; McLachlin and Miller, 1994). A subset of genes including *lef-1, lef-2, lef-3, lef-7, dnapol, p143, p35, ie-1* and *ie-2* were shown to participate in DNA replication (Kool *et al.*, 1994; 1995; Lu *et al.*, 1997; Lu and Miller, 1995). A further subset of genes, including *lef-4, lef-5, lef-6, lef-8, lef-9, lef-10, lef-11, lef-12, p47* and *pp31*, are required for late gene expression. Three other genes, *pk-1, pkip* and *vlf-1* are required for very late gene expression (Yang and Miller, 1998a,b; Rapp *et al.*, 1998; Fan *et al.*, 1998; Todd *et al.*, 1995; 1996; Fan *et al.*, 1996; McLachlin and Miller, 1994).

The role of some virus genes, such as *lef-2,* appears to be more complex than was first concluded. Originally, *lef-2* was assigned a role in virus DNA replication (Ahrens and Rohrmann, 1995; Lu and Miller 1995; Kool *et al.*, 1994). LEF-2 binds the *lef-1* product that carries a primase activity and could be involved in the initiation of DNA replication (Evans *et al.*, 1997). Recently, however, two independent approaches, have demonstrated that the *lef-2* product may be required for very late gene expression. A genetic approach showed that an AcMNPV mutant, VLD-1, with a mutation G:C to A:T transition in *lef-2*, was deficient in very late gene expression (Merrington *et al.*, 1996). Anti-sense inhibition of *lef-2* expression led to the same conclusion (Sriram and Gopinathan, 1998). Therefore LEF-2 could have a dual role in regulating both DNA synthesis and very late gene expression during virus replication.

To continue investigating the function of LEF-2, it would be interesting to remove *lef-2* from the virus genome and observe the effect on virus replication. The transient expression studies conducted by Passarelli and Miller (1993) suggest that such a virus would be non-viable.. Classical methods for deleting baculovirus genes using reporters such as *lacZ* are unsuitable for such a study, since the desired recombinant almost certainly cannot be propagated in insect cells. The failure to generate recombinant

viruses deficient in a particular gene often leads to the conclusion that the sequence is essential for virus replication. For example, the AcMNPV ORF1629 (Possee *et al.*, 1991) and a number of BmNPV genes (Gomi *et al.*, 1997). While undoubtedly correct in the majority of cases, it is also possible that an inability to isolate a mutant could be due to technical reasons.

Originally, the use of yeast to manipulate the baculovirus genome was designed to facilitate rapid production of recombinant virus (Patel *et al.*, 1992). Two separate elements are required for the stable propagation of a circular DNA molecule in yeast cells. An autonomously replicating sequence (ARS) is necessary to allow the DNA to be replicated independently of the yeast chromosome (Stinchcomb *et al.*, 1979). A CEN sequence is required to confer mitotic centromere function to the DNA molecule and ensure a stable low copy number in all transformed yeast cells (Fitzgerald-Hayes, 1987). A selectable marker is also necessary to identify yeast cells containing the baculovirus genome. In this study, URA3, which allows yeast cells to grow in the absence of uracil, was used. A second selectable marker, *sup4-o*, was also used for insertional inactivation of *lef-2*. This marker encodes a $tRNA^{Tyr}$ molecule, which suppresses ochre mutations of mutant yeast strains by insertion of a tyrosine residue at the UAA ochre nonsense codon (Goodman *et al.*,1977). The use of a suitable yeast strain containing ochre mutations in the *ade2* and *can1* genes enables selection of *sup4-o* positive colonies. Yeast ochre mutants in both *ade2* and *can1* grow as pink colonies in the absence of adenine and are insensitive to canavanine. Suppression of these mutations by *sup4-o* causes the production of white colonies that are sensitive to canavanine.

The inventors have inactivated the *lef-2* using yeast cells as an intermediary host to maintain the AcMNPV genome. The modified virus DNA was used to transfect insect cells to monitor the effect of the deletion on AcMNPV replication. It was also used to add a *c-myc* coding region to the 5' end of *lef-2*, which facilitated immunological detection of the protein in virus-infected cells.

This demonstrates that it is possible to generate and maintain replication deficient baculovirus in intermediate hosts. The virus can be rescued using a suitable rescue vector encoding a nucleic acid sequence to correct the deficiency. This has enabled a new method for cloning foreign genes in baculovirus to be identified.

The invention provides a method for cloning, and optionally expressing, a gene comprising the steps of:

(i) providing a replication deficient baculovirus vector;

(ii) providing a rescue vector encoding:

(a) a nucleic acid sequence which is capable of restoring replication in the replication-deficient baculovirus vector; and

(b) at least one gene to be cloned;

(iii) causing the replication-deficient baculovirus vector and rescue vector to recombine to produce a replication-enabled baculovirus vector comprising the at least one gene to be cloned; and

(iv) growing the replication-enabled baculovirus vector within a suitable invertebrate cell.

Preferably the invertebrate cell is an insect cell, but other suitable invertebrate cells in which baculovirus may grow may be used.

By replication-deficient baculovirus vector we mean a DNA molecule based upon the genome of a baculovirus, but which has had at least one gene necessary for replication either deleted or mutated so that the baculovirus vector can no longer replicate on its own. For example, one or more functional genes such as *lef-1, lef-2, lef-3, lef-4, lef-5, lef-6, lef-7, lef-8, lef-9, lef-10, lef-11, lef-12, dnapol, p143, p35, ie-1, ie-2, p47, ORF1629* and *pp31* may have been deleted or mutated to inactivate them.

The ability to replicate may be restored by recombination with a rescue vector comprising one of the functional genes, or a functional fragment or functional mutation thereof.

Preferably the baculovirus vector is based upon the genome of Ac MNPV.

By gene we mean a nucleic acid sequence which is capable of being transcribed into a protein or peptide of interest. The gene to be cloned is preferably operably linked to regulatory elements necessary for expression of said gene within the invertebrate cell. Operatively linked refers to the juxta position such that the normal function of the components can be performed. Control sequences refers to DNA sequences necessary for the expression of an operatively linked gene in a particular host organism. Control sequences which are suitable for eukaryotic cells for example are promoters, polyadenylation signals and enhancers. Preferably the control sequences include secretery signals to enable the product from the cloned gene to be secreted from the invertebrate cell.

Preferably the gene is under the control of a promoter selected from a baculovirus polyhedrin promoter and a baculovirus p10 promoter.

The advantage of the method of the invention is that it guarantees that substantially only recombinant virus containing the cloned gene is produced. This method avoids having to use time-consuming plaque assays or dot hybridisation to identify clones containing recombinant baculovirus. Accordingly, this allows the method to be automated using robotic devices and multi-well microtiter plates.

Preferably the replication-deficient baculovirus vector is capable of being maintained in an intermediate host. Such a host includes a yeast cell, such as *Saccharomyces cerevisiae* or a bacterial cell such as *Escherichia coli*. The precise strain of intermediate host selected will vary depending upon the construction of the replication-deficient baculovirus vector.

Preferably the replication-deficient baculovirus vector comprises one or more replication elements to enable replication within the intermediate host. For example, if the host is a yeast, the vector may comprise an autonomous replication sequence, such as ARS1 and a centromere functional sequence, such as CEN1. If the intermediate host

is a bacterial cell then the vector may comprise a bacterial origin of replication, *ori*. Such replication elements are well known in the art.

The replication-deficient baculovirus vector may also comprise one or more selection genes to enable host cells comprising the replication-deficient baculovirus vector to be selected. Such selection genes may be nutritional or antibiotic-resistance genes. For example, yeast nutritional genes such as URA3 or Sup4-0 may be used. Nutritional genes such as *Trp* and *His* may be used within bacteria. Similarly antibiotic-resistance genes such as ampicilin, tetracycline, Kanamycin or Neomycin resistance genes may be used. The host cell will be selected according to the selection gene used on the vector. For example, if URA3 is used as a selection gene, then the host cell will be one that cannot grow in the absence of Uracil unless the baculovirus vector containing URA3 is present. Cells containing the vector can then be selected by growing the yeast cells in the absence of Uracil. Alternatively, a bacterial cell comprising a replication-deficient baculovirus vector comprising an ampicillin resistance gene may be selected for by growing the cells in the presence of ampicillin.

The rescue vector may similarly contain replication elements to enable it to be replicated within a desired host, together with selection markers to enable cells containing the rescue vector to be identified.

The rescue vector preferably contains one or more sites into which a gene to be cloned may be inserted. Methods for inserting foreign genes into vectors are well known in the art. For example, the vector may comprise a unique restriction endonuclease site into which the gene of interest may be inserted. Preferably either side of the site is provided suitable promoter and termination sequences.

Rescue vectors which can insert multiple foreign gene coding sequences can be produced using techniques known in the art. For example, it is possible to construct a rescue vector comprising a polyhedrin gene promoter and transcription termination sequence upstream of, and in the opposite orientation to, a second polyhedrin gene promoter. It is also possible to insert a small DNA fragment encompassing the p10

promoter upstream, but in the opposite orientation of a polyhedrin promoter. The construction of multiple expression vectors is discussed in detail in the book by King and Possee (Supra).

The replication-deficient baculovirus vector and/or rescue vector may be inserted into a suitable intermediate host by techniques known in the art, such as lithium acetate (Ito *et al.* 1983) or calcium phosphate transfection (Graham and Van der Eb 1973, Wigler 1977).

The replication-deficient baculovirus vector and rescue vector may be recombined either within the intermediate host or most preferably within the invertebrate cell.

Typically, the replication-deficient baculovirus vector and the rescue vector are purified and isolated from any host in which they reside since purification techniques are well known in the art. For example, a total DNA preparation may be undertaken followed by sucrose or caesum chloride density gradient purification. Each of the vectors are inserted into a suitable invertebrate cell by known techniques, for example as shown in King and Possee (1992). The two vectors then undergo recombination within the cell to produce a replication enabled baculovirus vector comprising at least one gene to be cloned. Such recombined baculoviruses are selected for since they are able to undergo replication and thus infect further cells and multiply.

Preferably the replication deficient baculovirus vector lacks a gene coding for encoding polyhedrin. This has the advantage that contaminating polyhedrin is not produced.

The invention also relates to a replication-deficient baculovirus vector for use in a method of the invention. Preferably such a vector comprises one or more nucleic acid sequences which enable the vector to replicate within an intermediate host. Such sequences are described above.

A further aspect in the invention provides a rescue vector for use in a method according to the invention. Preferably the rescue vector is constructed as described above.

A still further aspect of the invention provides a kit for use in the method of the invention comprising a replication-deficient vector of the invention and/or a rescue vector according to the invention. The kit may additionally comprise one or more buffers, preservatives or stabilising agents to enable the vector(s) to be stored or transported with minimal degradation of the vector. Such buffers, preservatives and stabilising agents are well known in the art. The kit may also include instructions for carrying out a method according to the invention.

The invention will now be described by way of example only, with reference to the following figures:

Figure 1. Genomic organisation of parental and recombinant baculoviruses with modified polyhedrin and *lef-2*. (a) AcMNPV. Relative positions of *lef-2*, ORF603 and polyhedrin. (b) AcAUC. The insertion of the ARS-URA3-CEN4 cassette (not drawn to scale) relative to a deletion in polyhedrin. (c) AcΔ *lef-2.sup4-o*. The *sup4-o* was inserted into *lef-2* via homologous recombination in yeast cells harbouring AcAUC. Selected restriction enzyme sites are shown with appropriate genomic co-ordinates (Ayres *et al.*, 1994).

Figure 2. Genomic organisation of viruses with C-MYC added to LEF-2.
(a) Ac*lef-2.c-myc5'*. The c-myc coding region was inserted at the 5' end of *lef-2*. The C-MYC sequence is shown as bold text above the coding region. The additional amino acids introduced between C-MYC and LEF-2 via the XbaI site are italicised. (b) Ac*lef-2.c-myc3'*. The c-myc coding region was inserted at the 3' end of *lef-2*. The positions of oligonucleotide primers used to generate selected DNA fragments in the *lef-2* region for construction of the modified gene are indicated above and below the central genomic map.

Figure 3. Temporal expression of *lef-2* transcript.
(A) mRNA were extracted from *Sf*-21 cells either mock-infected (Mi), infected with *Acc-myclef-2* or AcMNPV C6 at a m.o.i. of 20, at the times (hours post infection)

indicated above the lanes. mRNA (100 ng) were subjected to RT-PCR using two internal specific primers of *lef-2*. The size of the specific RT-PCR product is indicated on the right. The W lane corresponds to the "smartladder SF" size marker from Eurogentec (1000, 800, 700, 600, 500, 400, 300, 200 and 100 bp from the top to the bottom).

(B) mRNAs were extracted from *Sf*-21 cells either mock infected (Mi) or infected with AcMNPV-C6 or *Acc-mycle f-2* at a m.o.i. of 20, at times (hours post infection) indicated above the lanes and subjected (2 µg per lane) to Northern blot. The sizes of the markers are shown on the left of the blot and the location of the major band hybridising with the *lef-2* probe is indicated by an arrow on the right.

Figure 4. 5' mapping of the *lef-2* transcripts.

Total RNA from mock infected or *Acc-mycle f-2*-infected cells were extracted at different times post infection and incubated with primer *lef-2* PE4 (A) or *lef-2* PE3 (B) depending on the promoter mapped (early or late). The reaction products were separated on a 6% sequencing gel, in parallel with a sequencing ladder generated with the same primer. Products are indicated on the right by arrows. Sequence and initiation sites are shown on the left. 12a: treatment with aphidicolin. 12c: treatment with cycloheximide.

Figure 5. Expression of LEF-4, GP-64 and CHI in *Acc-mycle f-2* and AcMNPV C6-infected *Sf*-21 cells.

Western blot analysis of steady-state levels of selected early (LEF-4 and GP-64) and late (GP-64 and chitinase) proteins in *Acc-mycle f-2* and wt-infected *Sf*-21 cells from 4 to 24 hr p.i. The protein bands are indicated by arrows. The numbers above each lane indicate time post infection and the proteins analysed is indicated on the left.

Figure 6. Western blot analysis of tagged-*lef-2* product in *Acc-mycle f-2* or AcMNPV C6-infected *Sf*-21 cells.

Western blot analysis of the tagged-*lef-2* product in *Acc-mycle f-2* or AcMNPV-C6-infected *Sf*-21 cells from 4 to 24 hr p.i. C-MYCLEF-2 protein was identified using the monoclonal anti-C-MYC antibody (clone9E10) and detected with a chemoluminescent substrate. The corresponding times post infection (hr p.i.) are

indicated above the lanes (Mi, mock infected). Size standards are indicated on the left and immunoreactive proteins are shown by arrows.

Figure 7. Sub cellular localisation of C-MYCLEF-2 by indirect immunofluorescence. *Sf*-21 cells were mock-infected or infected with *Accmyclef-2* and stained both with anti-C-MYC antibody (clone9E10) and anti-mouse conjugated to TRITC. A) Background fluorescence on uninfected cells. B) Cells stained at 9 hr p.i. C) Cells stained at 24 hr p.i. Laser levels were equivalent between all panels.

MATERIALS and METHODS

Insect cells and viruses

Spodoptera frugiperda IPLB-*Sf*21 cells were propagated at 28°C in TC100 medium supplemented with 10% (v/v) foetal calf serum (FCS). *Ac*MNPV C6 and other recombinant viruses were amplified and titrated according to standard protocols (King and Possee, 1992). *Ac*MNPV DNA replication was blocked by treating the virus-infected cells with aphidicolin (5 μg/ml; Sigma, USA) while protein synthesis was inhibited by using cycloheximine (200 μg/ml; Sigma, USA) according to a recent modification (Ross and Guarino, 1997).

Yeast cells and transformation

The *Saccharomyces cerevisiae* strain used in this study was y657 (*mat*α *his*3-11, 15 *trp*1-1 *ade*2-1 *leu*2-3, 112 *ura*3-52 *can*1-100 *his*4:HIS3; Newman and Norman, 1991) kindly provided by G. Patel, ICRF, London, UK. This was grown in rich medium (yeast extract, peptone, dextrose [YPD]) at 30°C. Yeast strains maintaining the baculovirus genome were grown in minimal medium supplemented with adenine (20 μg/ml), histidine (20 μg/ml), leucine (60 μg/ml), tryptophan (20 μg/ml) and casamino acids (10mg/ml) (Sherman, 1991). Canavanine resistant colonies were selected on minimal medium plates containing 60 μg/ml canavanine sulphate (Sigma, UK).

Transformation of yeast with the *Ac*MNPV genome and other plasmids was carried out with the lithium acetate method (Ito *et al.*, 1983) using high molecular weight DNA as a

carrier (Schiestl and Gietz, 1989). Briefly, y657 cells were grown in 5ml YPD medium to saturation. This culture was used to inoculate 250ml YPD medium, supplemented with 30 μg/ml adenine hemisulphate and the cells grown overnight at 30°C to a cell density corresponding to OD_{600}= 0.3 to 0.5. The cells were harvested by centrifugation (4000*g*; 5 min) and washed with 10ml sterile water. The cells were then resuspended in 1.5ml freshly prepared lithium solution (10mM Tris-HCl, pH7.5/1mM EDTA/0.1M lithium acetate, pH7.5). To transform the yeast cells, 200 μg carrier DNA (salmon sperm DNA boiled 3', then snap-chilled) was mixed with 5 μg transforming DNA in a volume of less than 20 μl. The yeast suspension (200 μl) was added to the DNA along with 1.2ml freshly prepared PEG solution (40% PEG 3350/ 10mM Tris-HCl, pH7.5/ 1mM EDTA/ 0.1M lithium acetate, pH7.5). The mixture was shaken at 30°C for 30 minutes, then heat-shocked at 42°C for exactly 15 minutes. The cells were pelleted and resuspended in 1ml TE buffer (10mM Tris-HCl, pH7.5/ 1mM EDTA). Aliquots (200 μl) were spread onto minimal media plates and incubated at 30°C for 3-4 days.

Plasmid and recombinant virus constructions

The plasmid p*Ac*AUC, containing the *ARS* (A), *URA3* (U) and *CEN* (C) sequences at the *polh* locus (Patel *et al.*, 1992) was mixed with *Bsu*36I-linearised BacPAK5 DNA (Kitts and Possee, 1993), in the presence of lipofection and used to transfect *Sf*21 cells (King and Possee, 1992). Progeny virus was plaque purified and amplified to produce polyhedrin-negative stocks (AcAUC) containing the yeast AUC elements. Infectious virus DNA was purified and used to transform y657 cells to yield yAcAUC.

The vector pUC8/6/8 (Possee, 1986), containing the AcMNPV *Eco*RI-I region was used to generate a plasmid containing a modified *lef-2* and the *sup4-o* yeast selectable marker. An octanucleotide *Bgl*II linker was inserted at the *Eco*RV site upstream of *polh* to create pUC8/6/8-*Bgl*II. This plasmid was digested with *Hind*III, recircularised and amplified in bacteria, then digested with *Sal*I (Fig. 1). The ends of the linear DNA were repaired with the Klenow fragment of DNA polymerase and ligated with a blunt-ended DNA fragment containing *sup4-o* to produce *plef2Δsup4-o*. (The *sup4-o* was originally obtained from G. Patel and inserted into the *Eco*RI site of pUC118 to provide a convenient supply). Lithium acetate-treated yAcAUC yeast cells were transformed

with *plef2Δ.sup4-o* and grown on minimal medium plates lacking uracil and adenine. Small white colonies were replica plated on the same medium in the presence or absence of canavanine sulphate. Canavanine sensitivity indicated that *sup4-o* was inserted into the virus genome maintained within the yeast cell.

To construct transfer vectors containing a c-myc epitope tag at either end of *lef*-2, oligonucleotides *c-myc*1 and *c-myc*2 were constructed (Table 1). These specified the sense and anti-sense *c-myc* sequences respectively. The oligonucleotides were annealed to create the double stranded *c-myc* coding region with *Bam*HI and *Pst*I cohesive ends. The epitope coding region also included an *Xba*I site directly upstream of the stop codon. The annealed oligonucleotides were inserted into a modified pUC118 plasmid (containing a *Bgl*II site directly upstream of the *Bam*HI site) between the *Bam*HI and *Pst*I sites to derive pUC.*c-myc*. Copies of the *lef*-2 coding region were created using PCR. These possessed either 5'-*Bam*HI and 3'-*Bgl*II ends and no TGA stop codon, or 5'-*Xba*I and 3'-*Pst*I ends including the TGA stop codon. These were inserted into pUC.*c-myc* separately at the *Bam*HI or the *Xba*I and *Pst*I sites respectively to derive two plasmids in which the *c-myc* epitope was either upstream or downstream of *lef*-2. PCR was then used to derive upstream and downstream regions of *lef*-2 possessing 5'-*Kpn*I, 3'-*Bgl*II or 5'-*Pst*I, 3'-*Hin*dIII ends respectively. These were inserted at either end of the *c-myc*-tagged *lef*-2 in both plasmids to derive p*Aclef*-2.*c-myc*5' and p*Aclef*-2.*c-myc*3' (Fig. 2).

Yeast DNA extraction and transfection of insect cells

Total yeast DNA was prepared as described (Patel *et al.*, 1992) and layered onto a 5-20% continuous sucrose gradient in STE (200mM NaCl/ 10mM Tris pH7.5/ 1mM EDTA). The gradient was centrifuged at 35,000 rpm for 3 hours at 20°C in a Beckman SW41 rotor. The gradient was then harvested in 0.5ml fractions by downward displacement. The DNA in each fraction was ethanol precipitated, pelleted and resuspended in 50 µl TE pH 7.5. Aliquots (10 µl) of each DNA fraction were placed in sterile polystyrene bijou bottles with 0.5ml TC100 lacking FCS; on occasion, 500 ng of a rescuing plasmid (pUC8/6/8) was included. An additional 0.5ml TC100 minus FCS containing 5 µl lipofectin was added to each DNA solution and the mixtures were

incubated at room temperature for 15 to 30 minutes. Thereafter, 3 x 10^5 *Sf*21 cells in 35mm cell culture dishes were transfected as described by King and Possee (1992). The dishes were incubated at 28°C for 6 days and monitored daily for signs of viral infection. The virus-containing medium was then harvested and titrated in a plaque assay.

Western blot and immunofluorescence analysis

Monolayers of 10^7 *Sf*21 cells were infected with *Ac*MNPVC6 or *Aclef-2.c-myc5'* at a multiplicity of infection (m.o.i.) of 20 or mock-infected with medium. Cells were harvested at various times, pelleted, washed twice with PBS and lysed in dissociation mixture (Laemmli, 1970). Protein samples (equivalent of 3 x 10^6 cells) were then loaded and separated by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were incubated overnight in Tris-NaCl buffer (TBS: 137mM NaCl; 20mM Tris-HCl, pH 7.6) containing 10% dried milk and 0.1% Tween-20. C-MYC monoclonal (clone 9E10), GP64-EFP monoclonal (AcV5), LEF-4 polyclonal and CHI [*Ac*MNPV chitinase] polyclonal antisera have been described previously (Evan *et al.*, 1985; Blissard and Rohrmann, 1989; Durantel *et al.*, 1998a; b; Hawtin *et al.*, 1995, respectively).

Western blots were performed with appropriate dilutions of polyclonal or monoclonal antibodies. Immunoreactive proteins were detected using the appropriate secondary antibody linked to the peroxidase (Sigma, USA) followed by incubation with a chemoluminescent substrate (Amersham, UK), according to the manufacturer's instructions.

Immunofluorescence staining was performed as described previously (Durantel *et al.*, 1998b) with an important modification. First and secondary antibodies were applied twice in order to amplify the staining. The preparations were examined under a Zeiss LSM410 confocal laser scanning microscope.

RNA pr cedures

Messenger RNA isolation, Northern blot, primer extension, RT-PCR and 3'RACE (rapid amplification of cDNA ends) were described previously (Durantel *et al.*, 1998a; b). Extraction of total RNA was performed using the RNeasy midi kit following the manufacturer's instructions (Qiagen, USA). The synthetic oligonucleotides used in primer extension were lef-2PE3 and lef-2PE4; in RT-PCR, lef-2RT-1 and lef-2stop; in RT-PCR, lef-2RT1 and oligo(dt). The sequence of each primer is listed in Table 1, with further information on its position relative to the AcMNPV genome

RESULTS

Production of a recombinant baculovirus DNA containing a deletion within the lef-2 gene using yeast cells as intermediate host.

A recombinant virus (AcAUC), containing the *ARS, CEN* and *URA3* sequences at the *polh* locus, was produced to enable propagation of viral DNA in yeast cells. The genetic organisation of this virus is shown in Figure 1. The correct insertion of the three yeast elements in the *Ac*MNPV genome was confirmed by Southern hybridisation analysis (data not shown).

Virus DNA was extracted from purified AcAUC budded virus particles and used to transform the yeast strain y657. Yeast cells containing the AcAUC DNA were selected on dropout plates lacking uracil. Yeast cells were transformed at an efficiency of approximately 2 x 10^5 colonies per µg transforming DNA. Total DNA was extracted from amplified yeast cultures (yAcAUC), fractionated on sucrose gradients and used to transfect *S. frugiperda* cells. After six days, it was determined that the transfected cell culture medium contained 5 x 10^4 pfu/ml. Cell cultures transfected with 100 ng AcMNPV DNA produced 9.7 x 10^5 pfu/ml over the same period. Subsequent amplification of AcAUC derived from yeast in insect cells produced virus stocks of normal titre (> 10^7 pfu/ml).

In order to delete *lef-2* from *Ac*AUC DNA maintained in y*Ac*AUC, we produced a transfer vector (p*lef-2_sup4-o*) containing *sup4-o* in lieu of *lef-2* (Fig. 1c). Yeast strains

with ochre mutations in both *ade2* and *can1* grow as pink colonies in the absence of adenine and are insensitive to canavanine. Suppression of these mutations by *sup4-o* causes the production of white colonies that are sensitive to canavanine. The yAcAUC cells were transformed with plef-2.*sup4-o* and colonies containing *sup4-o* were selected by growth on dropout plates lacking uracil and adenine. Adenine independent colonies (white) were then replica-plated onto dropout plates lacking uracil but containing canavanine sulphate. Colonies sensitive to canavanine were retro-selected. Twelve yeast colonies were isolated with the correct phenotype of *ura3+* and *sup4-o+* by replica plating and amplified in liquid culture.

Transfection of insect cells with viral DNA isolated from *ura3+*, *sup4-o+* yeast colonies.

Virus DNA was isolated from twelve yeast clones identified as *ura3+* and *sup4-o+*. Each sample was used to transfect *S. frugiperda* cells in the presence or absence of pUC8/6/8, a plasmid containing an intact *lef-2* (Possee *et al.*, 1991). The addition of this plasmid to the transfection mix was predicted to rescue the expected deletion in *lef-2*, via homologous recombination in insect cells, and permit virus replication. Nine out of the twelve DNA preparations caused infection of the *S. frugiperda* cells irrespective of the presence of the rescuing plasmid (data not shown). When each of the other three DNA preparations (AcΔ*lef-2.sup4-o*^{9}, ^{12} or ^{21}) were used to transfect *Sf*21 cells without pUC8/6/8, productive infection of the cells was not observed. However, co-transfection of insect cells with each of these DNA preparations and the rescuing plasmid resulted in low levels of infectious budded virus production (Table 2). In comparison with insect cells transfected with 100 ng AcMNPV DNA, 33 - 200-fold less virus was produced. When these primary virus stocks were amplified in suspension cultures of insect cells, however, normal virus tires were attained for all samples (Table 2).

Production of a c-myc-tagged AcMNPV lef-2

The AcΔ.*lef-2.sup4-o*^{9} was used to derive a recombinant virus with a human C-MYC coding region added to that of *lef-2*, to permit the identification of LEF-2 in virus-infected cells. The C-MYC epitope or tag comprises 12 amino acids (NH_2 -MEQKLIEEDLNSR-COOH), which can be recognised by a monoclonal antibody (9E10; Evan *et al.*, 1986). It was not known whether addition of this epitope would affect the folding and/or the activity of LEF-2 when added to either end of the protein. Two transfer vectors were constructed containing the *c-myc* coding sequence at either the 5' (p*Aclef-2.c-myc5'*) or 3' (p*Aclef-2.c-myc3'*) ends of the *lef-2* coding region (Fig. 2). These were used individually to co-transfect insect cells with AcΔ.*lef-2.sup4-o*^{9} DNA isolated from yeast cells. After 6 days, the cell culture medium for each sample was titrated in a plaque assay to monitor virus production. Infectious virus (Aclef-2.5'c-myc) was only produced by cells co-transfected with AcΔ.*lef-2.sup4-o*^{9} and p*Aclef-2.c-myc5'*. This was amplified further in insect cells to derive a virus stock (*Aclef-2.c-myc5'*) of comparable titre to that produced by AcMNPV (ca. 1-2 x 10^8 p.f.u./ml)

To confirm that the *c-myc* coding region was present at the 5' end of *lef-2* in *Aclef-2.c-myc5'*, total DNA was extracted from virus-infected cells and the *lef-2* region amplified by PCR. The resulting DNA fragment was digested with *Bam*HI to detect the extra site inserted at the start of *lef-2* when *c-myc* was present. All three viruses exhibited the presence of c-myc at the 5'-end of *lef-2* (data not shown).

Analysis of lef-2 transcription

The production of *Aclef-2.c-myc5'* virus stocks of normal infectivity suggested that transcription of *lef-2* was not affected by the addition of the *c-myc* sequences. This was confirmed by analysing the temporal regulation of *lef-2* using RT-PCR to amplify purified mRNA extracted from AcMNPV-, *Aclef-2.c-myc5'*- or mock-infected cells. Two oligonucleotide primers (*lef-2*RT1 and *lef-2*stop) internal to *lef-2* were used to amplify a 283-bp fragment. For both virus infections, *lef-2*-specific mRNA sequences were amplified as early as 1 hr p.i., with yields increasing until 12 h p.i. The transcripts remained stable until at least 48 hr p.i. (Fig. 3A). The *lef-2* transcripts were also

analysed at selected times post infection using Northern blot hybridisation with a strand-specific riboprobe. Figure 3B shows a typical result obtained with AcMNPV. A major transcript with a size of 1 kb appeared weakly at 5 hr p.i. and accumulated to a higher level up to 24 hr p.i. Other products including a band between 1.5 and 1.6 kb were detected both at early and late times after infection (Fig. 3B). Similar results were obtained with Ac*lef-2.c-myc5'*.

The transcription starts sites for *lef-2* in AcMNPV-infected cells were determined by primer extension using two different oligonucleotides. Primer *lef-2*PE4 was used to detect the early start site. An extension product was obtained with mRNA extracted at 6 and 12 hr p.i., corresponding to a start site located 279 bp upstream from the *lef-2* ATG codon. This mapped at a C within the sequence CAATGCGCCCGTTGT (Fig. 4A). When virus-infected cells were treated with cycloheximide, no products were obtained in the subsequent primer extension analysis, underlying the early character of this promoter. Primer *lef-2*PE3 was used to detect the late start site. One major extension product was obtained with mRNA extracted at 12 and 24 hr p.i. (Fig. 4B). This mapped the transcription start site at an adenine within a TAAG motif located 361 bp upsteam of the ATG. When mRNA was extracted from virus-infected cells treated with aphidicolin, no primer extension products were obtained. Identical results were obtained with mRNA from Ac*lef*-2.*c-myc5'* -infected cells (data not shown).

The 3' end of the *lef-2* transcript was determined by sequencing DNA fragments generated by RACE-PCR after RT amplification of mRNA purified at 9 hr p.i. from AcMNPV- or Ac*lef*-2.*c-myc5'*-infected cells. A single product with a length of approximately 330 bp was obtained from both samples (data not shown). Using the *lef-2* RT1 primer, this fragment was sequenced to determine the precise point of addition of the poly(A) tail. This mapped at a A residue located 17 bp and 9 bp downstream from the stop codon of *lef-2* and a AATAAA motif respectively. Combining the data from primer extension and 3' end mapping predicts an early *lef-2* specific transcript of 928 bases and a late transcript of 1000 bases in AcMNPV-infected cells.

Expression of LEF-4, GP-64 and CHI in Ac*lef*-2.*c-myc5'* -infected cells

To confirm that synthesis of a modified form of LEF-2 did not affect expression of other virus genes, the production of three other virus proteins was examined. These included an early gene (*lef-4*), a gene with both early and late transcription start sites (*gp67*) and a gene with only a late promoter (*chiA*). When production of these proteins was compared in Ac*lef*-2.5'*c-myc*- or AcMNPV-infected cells, no differences in temporal synthesis or accumulation were observed (Fig. 5). It was interesting to note, however, that LEF-4 declined in abundance from about 12 h p.i.

Immunodetection of the C-MYC-tagged-LEF-2 in infected cells

Insect cells infected with Ac*lef*-2.*c-myc5'* were examined using Western blot analysis in conjunction with the C-MYC-specific monoclonal antibody (9E10) to detect the tagged-LEF-2 (Fig. 6A). A specific product of approximately 25 KDa was detected in Ac*lef*-2.*c-myc5'*-infected cells. The size of this product was consistent with the predicted combined molecular weights of LEF-2 (23.9 kDa; Ayres *et al.*, 1994) and C-MYC (1.6 kDa). The protein was first detected weakly at 4 h p.i. and increased in abundance until 24 h p.i., the last point tested. In *Ac*MNPV-infected cells, the 25 kDa protein was not detected (Fig. 6B). In both experiments a major extra band, around 35 KDa, was detected even in the mock-infected lane. This corresponded to a cross reaction of our antibody against a host protein.

It was predicted that LEF-2 would be localised in the nuclei of virus-infected cells. It is associated with both DNA replication and late gene transcription. Both biochemical fractionation and immunofluorescence microscopy were used to test this hypothesis. Only the confocal microscopy observations are shown in Figure 7. Staining was found mainly in the nucleus of infected cells at both 9 and 24 hr p.i. The staining appeared to be concentrated in a central area that seemed to match the virogenic stroma. At early times after virus-infection (9 hr p.i.), the staining was observed as small discrete areas (foci) in the nucleus. Later in virus infection (24 hr p.i.) a large central area was

stained. These results indicated that LEF-2 localised in the nucleus at both early and late times post infection.

DISCUSSION

In this study, we used *S. cerevisiae* as a host to maintain the AcMNPV genome and permit the manipulation of *lef-2*, a gene that is essential for replication of the virus in insect cells (Passarelli and Miller, 1993). The method was adapted from a protocol developed for the production of recombinant viruses containing foreign genes, that eliminated the need for plaque purification (Patel *et al.*, 1992). We retained the AUC elements, inserted in lieu of the AcMNPV polyhedrin gene, to facilitate DNA replication in yeast cells. Part of the *lef-2* coding region was replaced with *sup4-o* within a transfer vector. This plasmid was then introduced into yeast cells harbouring the AcMNPV genome to effect the modification of *lef-2* via homologous recombination. Although *lef-2* was targeted in this study, the method could be readily adapted to modify any baculovirus gene. It should circumvent the difficulties frequently encountered when trying to inactivate virus-coding regions by insertion of a reporter gene such as *lacZ* after cotransfection of insect cells with virus DNA and a transfer vector. If the target sequence is essential for virus replication, recombinant viruses are unstable and rapidly lost from the population. This problem was encountered when trying to modify the AcMNPV ORF1629 (Possee *et al.*, 1991), which encodes a nucleocapsid-associated protein (Vialard and Richardson, 1993). The failure to isolate a recombinant virus was indicative, but not satisfactory evidence that this gene cannot be deleted from the virus genome.

The product of *lef-2* (Passarelli and Miller, 1993) has a key role in viral replication. It was originally shown to be required for DNA replication (Kool *et al.*, 1994; 1995; Lu and Miller, 1995; Lu *et al.*, 1997). This conclusion derived from experiments where the transient replication of a reporter plasmid in the presence or absence of a number of baculovirus genes was measured. Although a very powerful system, its design precludes the identification of other roles for these genes in events subsequent to DNA replication. In other studies it was suggested that LEF-2 could also be involved directly

in the regulation of very late gene expression, via the use of a virus with a mutation within the gene (Merrington *et al.*, 1996) or anti-sense inhibition of its transcription (Sriram and Gopinathan, 1998). Clearly, there is scope for alternative approaches to studying the functions of this protein.

The recombinant virus DNA (AcΔ*.lef-2.sup4-o*) from yeast, with a deletion in *lef-2*, was unable to transfect *Sf*21 cells. This provided unambiguous evidence that *lef-2* is required for propagation of the virus in insect cells. However, co-transfection of this DNA with a plasmid containing an unmodified copy of *lef-2* permitted the recovery of infectious virus. This showed that no other mutation within the AcΔ*.lef-2.sup4-o* genome was responsible for the failure of the virus to replicate in insect cells. In future studies, it should be possible to co-transfect insect cells with AcΔ*.lef-2.sup4-o* DNA and plasmids containing selected modifications to *lef-2*. This would enable the function of each domain of the protein to be dissected *in vivo*, as judged by the failure or otherwise to regenerate an infectious virus. Incorporating a suitable reporter gene under early or late promoters into the system would also allow direct measurements of virus gene expression at different times after infection.

Some technical problems were noted during the use of the yeast system. Viral DNA extracted from yeast was not very efficient at establishing an infection when used to transfect *Sf*21 cells. Once the primary infection was established, however, virus stocks of normal titre could be obtained after subsequent passage in the same cells. This is probably due to the procedure used to extract the DNA from the yeast cells. The method is protracted and employs phenol extraction and ethanol precipitation to purify total DNA from the cells. It has previously been noted that precipitation of viral DNA reduces infectivity, due to the inevitable shearing that results on resuspension (King and Possee, 1992). The low infectivity of the viral DNA from yeast may, therefore, be due to only a subpopulation of the DNA remaining as intact circles.

A low number of yeast colonies were obtained on transformation of the strain carrying the intact *Ac*MNPV genome with the transfer vector containing the *sup4-o* gene. This may have been due to either a low recombination frequency or the action of the *sup4-o*

gene product itself. When the yeast cells were transformed with the *sup4-o*-containing transfer vector, recombination was required to permit insertion of the *sup4-o* gene in place of *lef*-2. The efficiency of this recombination has not been estimated in yeast cells. It has been noted that, in insect cells, recombination occurs at a frequency of between 0.1 and 1%. A low frequency of recombination would lead to the production of only a few *sup4-o*+ colonies. Alternatively, the *sup4-o* gene encodes an ochre-suppressing tRNA. The ochre suppression may be a more stringent selection than an auxotrophic marker. We are currently investigating the use of alternative markers as insertable elements.

It was found that nine out of the twelve *sup4-o*-containing colonies produced virus DNA that was able to infect insect cells irrespective of the presence of the rescuing plasmid. This may have been due to the *sup4-o* gene inserting at alternate loci in the viral genome, causing no effect on virus replication. The *sup4-o* gene may also have inserted into the yeast chromosome itself, as it is known that only very short sequences are required for homologous recombination in yeast. We are currently working to improve the number of positive recombinants obtained.

We also used AcΔ*.lef*-2*.sup4-o* DNA as an intermediate step in the construction of a virus with an antigenic tag at the extremities of the protein. Tags are short polypeptide sequences for which we possess powerful monoclonal antibodies that facilitate identification and purification of the protein, even if it is present in low levels in the cells. In this example, the tagging procedure was successful only at the N terminal of LEF-2, suggesting that the C-terminal end is a critical position for the function of the protein. Recently, the Asp residue (D178) in the C-terminal of the protein was associated with the regulation of very late gene expression (Merrington *et al.*, 1996). In addition, it has been noticed that the C-terminal domain is a cysteine rich area that presents structural homology with proteins, such as Adenovirus p300/CBP, involved in gene regulation (Eckner et al., 1994). Therefore it seems likely that modification of the C terminal end of LEF-2 cannot be tolerated because of a crucial role in its function.

Gene expression was compared in cells infected with AcMNPV or *Aclef-2c-myc5'*, to determine if the addition of the tag to LEF-2 had any effect on transcription. Transcription analysis of *lef-2* in AcMNPV and *Aclef-2c-myc5'*-infected cells showed no differences between the two viruses. In both viruses, a dual promoter included within the 350 nucleotides upstream of the ATG drove the expression of *lef-2*. The *lef-2* transcripts were detected as early as 1 hr p.i. and reached a high level by 48 hr p.i. The early start site was mapped at a C within the sequence CAATGCGCCCGTTGT localised 279 nt upstream of the *lef-2* ATG. A TATA-like sequence was evident between 35 and 30 nucleotides upstream from the transcription initiation site, confirming the cellular structure of this promoter. The late start site was mapped within the characteristic sequence TAAG located 361 nucleotides upstream of the start codon. Similar sites were recently found for the closely related BmNPV *lef-2* (Sriram and Gopinathan, 1998). Identifying the polyadenylation anchor site 17 nucleotides downstream from the lef-2 translation stop codon completed the transcriptional analysis. The production of LEF-4, GP64 and chitinase was studied to determine that the addition of the C-MYC tag to LEF-2 did not have subtle effects on early and late virus gene expression. We concluded that the modification to *lef-2* was neutral with respect to virus replication.

Using an antibody against the C-MYC epitope, the kinetics of LEF-2 production was analysed. The protein, with an expected size of 25 kDa, was first detected four hours after infection. Its quantity increased during the late phase of the infection. This result was in agreement with the RNA concentration during the viral cycle. However, the level of expression was found to be quite low even though a late promoter was involved in the transcription of the *lef-2*. Recent data suggested the importance of the sequence directly upstream from the TAAG motif for the selection and the level of expression of a late promoter (Mans and Knebel-Mörsdorf, 1998). Reduced helix stability was found to correlate with functional TAAG motifs. In the case of the *lef-2* late promoter, the upstream sequence was sufficiently A/T-rich to create a functional late promoter but not high enough to derive a strong late promoter. This observation may explain the results obtained for the LEF-2 production. Due to the low level of *lef-2* expression, the detection of the protein by a classic indirect immuno-fluorescence was impossible with

our anti-c-myc antibody. We improved the sensitivity of detection using an amplification method which consisted of incubating fixed cells twice with primary and secondary antibodies. We observed a nuclear localisation of LEF-2 at both early and late times post infection. This was consistent with roles for the protein in both DNA replication and late virus gene expression. Others antibodies obtained against the complete LEF-2 over produced in bacteria or against synthetic peptides failed to detect efficiently the protein in both western blot and indirect immunofluorescence microscopy, revealing the poor antigenic character of the protein. These results, or lack of them, validate the approach of using a well-defined epitope and appropriate antibody for the detection of such proteins. The method should be applicable to any protein that is expressed at a low level in baculovirus-infected cells and presents difficulties in raising an antiserum.

The results show that it is possible to rescue replication deficient virus using a rescue vector encoding a gene encoding a protein for restoring replication. The inventors have realised that it is possible to take this a step further by inserting a foreign gene into the rescue vector. The foreign gene may then be recombined with the baculovirus at the same time as the gene for restoring replication. Such a recombinatorial event can be selected for because the recombined virus containing the foreign gene will be able to replicate.

This avoids the use of time consuming plaque assays or dot hybridisation procedures, previously known in the art, to identify virus containing foreign genes.

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Table 1.

Sequences of synthetic oligonucleotides.

Oligo	Sequence[a]	Strand[b]	REN site[c]	Position
*c-myc*1	GATCCATAATGGAGCAAAAGCTCATTTCTGAA GAGGACTTGAATTCTAGATAACTGCA	+	*Xba*I	N/A
*c-myc*2	GTTATCTAGAATTCAAGTCCTCTTCAGAAATGA GCTTTTGCTCCATTATG-	-	*Xba*I	N/A
CLH1	*CGGC*AGATCTATAATGGCGAATGCA	+	*Bgl*II	3089-3100
CLH2	*GCCA*GGATCCATAATTACAAATAGGATTGAG	-	*Bam*HI	3698-3718
CLH3	*CGGC*TCTAGAATGGCGAATGCATC	+	*Xba*I	3089-3102
CLH4	*GCCGC*TGCAGTCAATAATTACAAATAGG	-	*Pst*I	3704-3721
CLH5	*GGCCGGTACC*GAGTTCGTTGACGC	+	*Kpn*I	2334-2347
CLH6	*CGCGA*GATCTACTTCGCGGCTTCTCGCACC	-	*Bgl*II	3069-3088
CLH7	*GGCCCTGCAGA*TAATAAAACAATTATAAAT	+	*Pst*I	3722-3741
CLH8	*CGCGAAGCTTA*GCAACTATATATT	-	*Hin*dIII	4411- 4424
*Lef-2*PE3	AAGCTCGTGCCGGAACGCGTGCACAGATCG	-		2886-2915
*Lef-2*PE4	TGTAGTCGGCAGTTCATTTTGGGCGTGATCG	-		2966-2995
*Lef-2*RT1	AAGAAAACAATGTACCGCGCGGCGG	+		3438-3462
*Lef-2*stop	ATGCGAATTCTCAATAATTACAAATAGGATTG	-	*Eco*RI	3700-3721

[a] The first first nucleotide corresponding to AcMNPV sequence is shoewm
[b] Relative to the coding strand of *lef-2*.
[c] The restriction enzyme site is underlined in the sequence of the oligonucleotide.

Tabl

Transfection of *Sf*21 cells with virus DNA

Viral DNA	pUC8/6/8	Titre after transfection (p.f.u./ml)[a]	Titre after progeny virus amplification (p.f.u./ml)[b]
AcMNPV (100ng)	-	2×10^6	5×10^7
yAcΔ*lef-2.sup4-o*^{9}	-	ND	ND
yAcΔ*lef-2.sup4-o*^{9}	+	9×10^3	5×10^7
yAcΔ*lef-2.sup4-o*^{10}	-	ND	ND
yAcΔ*lef-2.sup4-o*^{10}	+	5×10^4	5×10^7
yAcΔ*lef-2.sup4-o*^{21}	-	ND	ND
yAcΔ*lef-2.sup4-o*^{21}	-	6×10^4	5×10^7

[a] Progeny virus was titrated 6 days after transfection of *Sf*21 cells.
[b] Infectivity of virus stocks after amplification in *Sf*21 cells.

CLAIMS

1. Method for cloning a gene comprising the steps of:

(i) Providing a replication-deficient baculovirus vector;

(ii) Providing a rescue vector encoding:

(a) a nucleic acid sequence which is capable of restoring replication in the replication-deficient baculovirus vector; and

(b) at least one gene to be cloned.

(iii) Causing the replication-deficient baculovirus vector and rescue vector to recombine to produce a replication-enabled baculovirus vector comprising the at least one gene to be cloned; and

(iv) Growing the replication-enabled baculovirus vector within a suitable invertebrate cell.

2. Method according to claim 2, wherein the invertebrate cell is an insect cell.

3. Method according to claim 1 or claim 2, wherein the replication-deficient baculovirus vector lacks a functional gene necessary for viral replication and the rescue vector comprises a gene necessary for restoring the functional gene.

4. Method according to claim 3, wherein the functional gene is selected from *let-1*, *lef-2*, *lef-3*, *lef-4*, *lef-5*, *lef-6*, *lef-7*, *lef-8*, *lef-9*, *lef-10*, *lef-11*, *lef-12*, *dnapol*, *p143*, *p35*, *ie-1*, *ie-2*, *p47*, *ORF1629* and *pp31*, or a functional fragment or mutation thereof.

5. Method according to claim 4, wherein the functional gene is *lef-2* or a functional fragment or mutation thereof.

6. A method according to any preceding claim wherein the replication deficient baculovirus vector is capable of being maintained in an intermediate host.

7. A method according to claim 6, wherein the intermediate host is a yeast cell or a bacterial cell.

8. A method according to claim 6 or claim 7, wherein the replication deficient baculovirus vector comprises one or more nucleic acid sequences which enable the vector to replicate within the intermediate host.

9. A method according to any preceding claim wherein the recombination step (iii) takes place within the invertebrate cell.

10. A method according to any preceding claim, additionally comprising the step of (v) growing invertebrate cell so that the or each foreign gene is expressed within the cell.

11. A replication deficient baculovirus vector for use in a method according to any preceding claim.

12. A vector according to claim 11, additionally comprising one or more nucleic acid sequences which enable the vector to replicate within an intermediate host.

13. A vector according to claim 12, wherein the vector comprises one or more nucleic acid sequences selected from ARS-1, CEN-1 and a bacterial origin of replication (*ori*).

14. A vector according to any one of claims 11 to 13, in which at least a part of the naturally occurring polyhedrin gene has been deleted.

15. A vector according to any one of claims 11 to 14, wherein the vector is based upon *Ac*MNPV.

16. A rescue vector for use in a method according to any one of claims 1 to 10 comprising (a) a nucleic acid sequence which is capable of restoring replication in a replication-deficient baculovirus, and (b) at least one site for insertion of a gene to be cloned.

17. A rescue vector according to claim 16, wherein the site for insertion of the gene to be cloned is operably linked to regulatory elements necessary for expression of said gene.

18. A rescue vector according to claim 17, wherein the site for insertion of the foreign gene is operably linked to a promoter selected from a baculovirus polyhedrin promoter or a baculovirus p10 promoter.

19. An intermediate host cell comprising a replication-deficient baculovirus vector according to any one of claims 11 to 15.

20. A cell comprising a rescue vector according to any one of claims 16 to 18.

21. An invertebrate cell obtainable by a method according to any one of claims 1 to 10.

22. A kit for use in a method according to any one of claims 1 to 10 comprising a replication deficient vector according to any one of claims 11 to 15.

23. A kit according to claim 22 additionally comprising a rescue vector according to any one of claims 16 to 18.

24. A kit for use in a method according to any one of claims 1 to 10 comprising a rescue vector according to any one of claims 16 to 18.

25. A kit according to any one of claims 22 to 24 additionally comprising one or more buffers, preservatives or stabilising agents.

ABSTRACT

BACULOVIRUS EXPRESSION SYSTEM

The application relates for method for cloning the gene comprising the steps of:

1. Providing a replication-deficient baculovirus vector;

2. Providing a rescue vector comprising:

(a) nucleic acid sequence which is capable of restoring replication in the replication-deficient baculovirus vector; and

(b) at least one gene to be cloned;

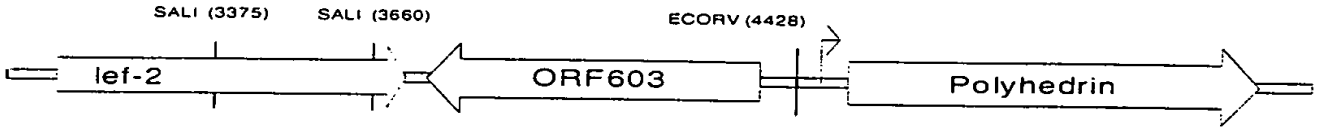
3. Causing the replication-deficient baculovirus vector and rescue vector to recombine to produce a replication-enabled baculovirus vector comprising the at least one gene to be cloned; and

4. Growing the replication-enabled baculovirus vector within a suitable invertebrate cell, such as an insect cell. Preferably the baculovirus vector is based upon *Ac*MNPV.

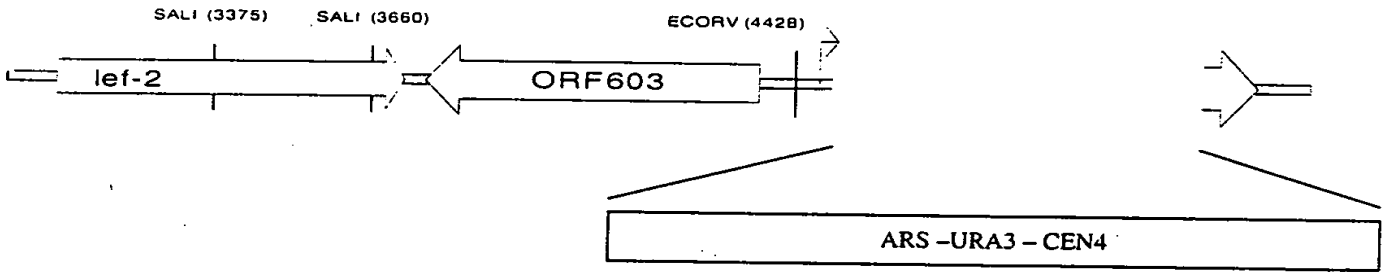
Also disclosed are replication-deficient baculovirus vectors, rescue vectors, cells containing such vectors and kits comprising such vectors.

Figure 1

(a) AcMNPV



(b) AcAUC



(c) AcΔlef-2.*sup4-o*

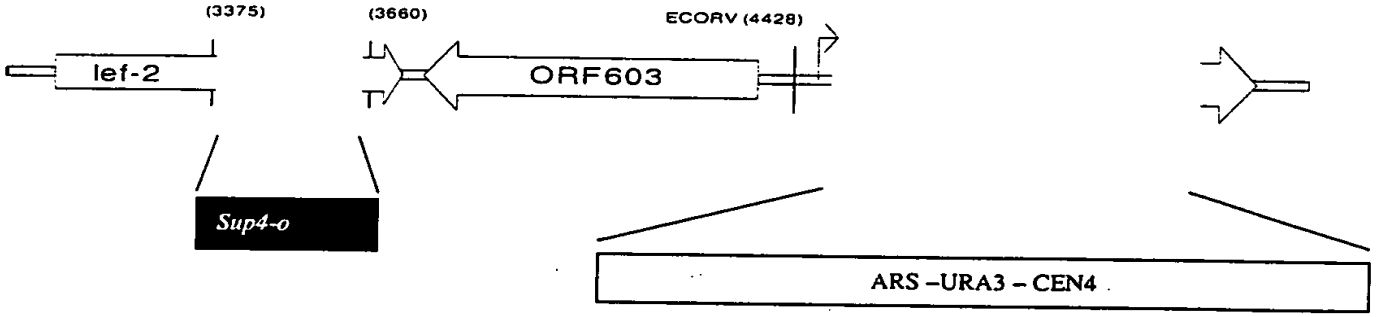
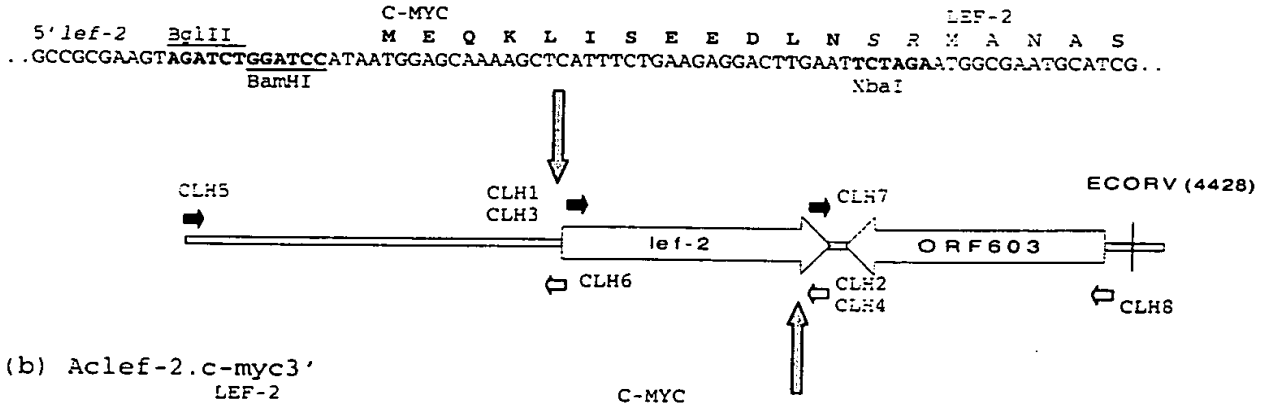


Fig 2

(a) Aclef-2.c-myc5'



(b) Aclef-2.c-myc3'

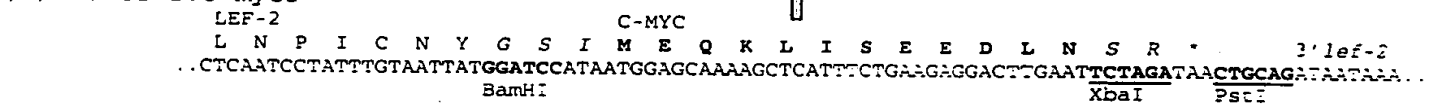


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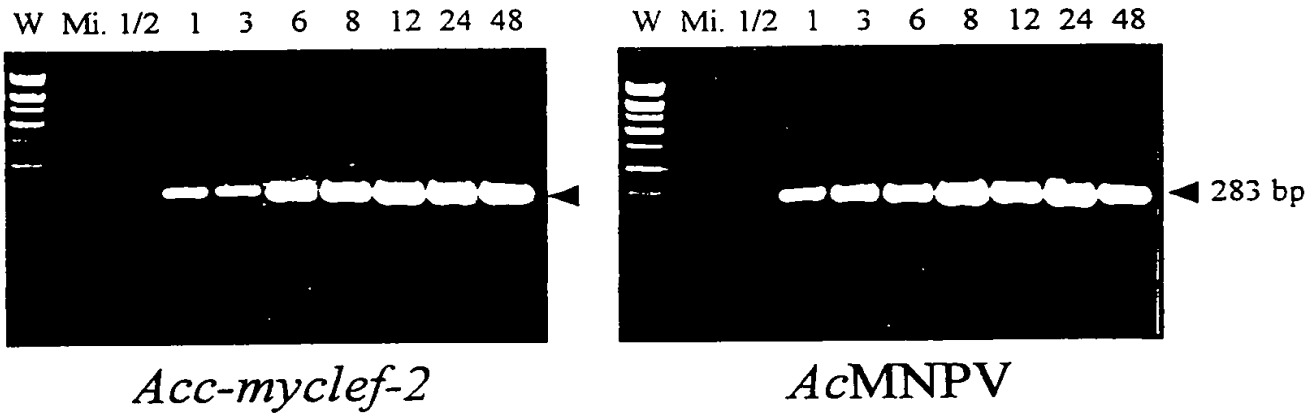
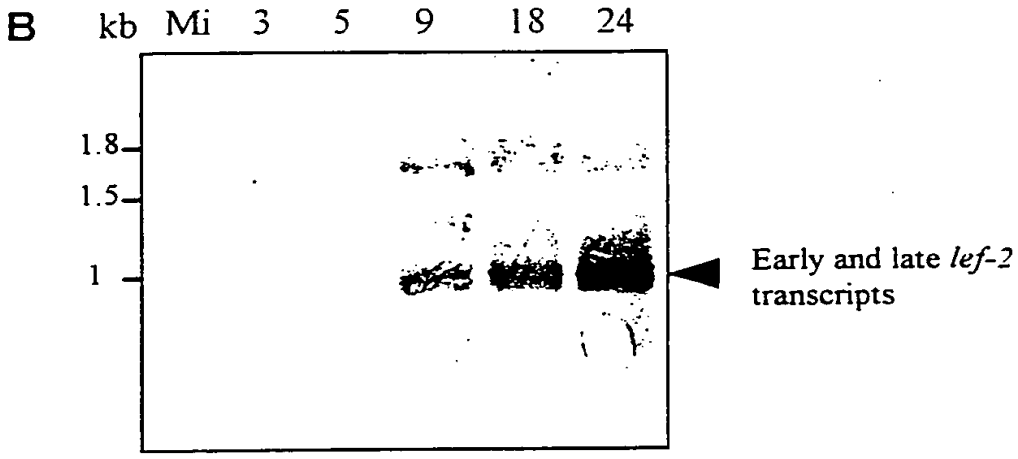
Figure 3



Acc-myclef-2 *Ac*MNPV



Acc-mvclef-2 or *Ac*MNPV

Figure 4

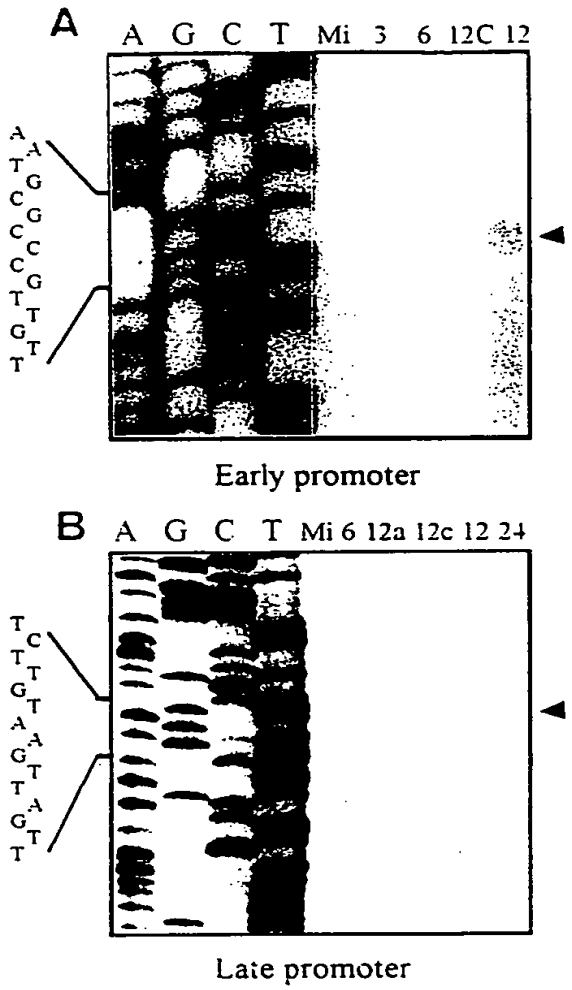
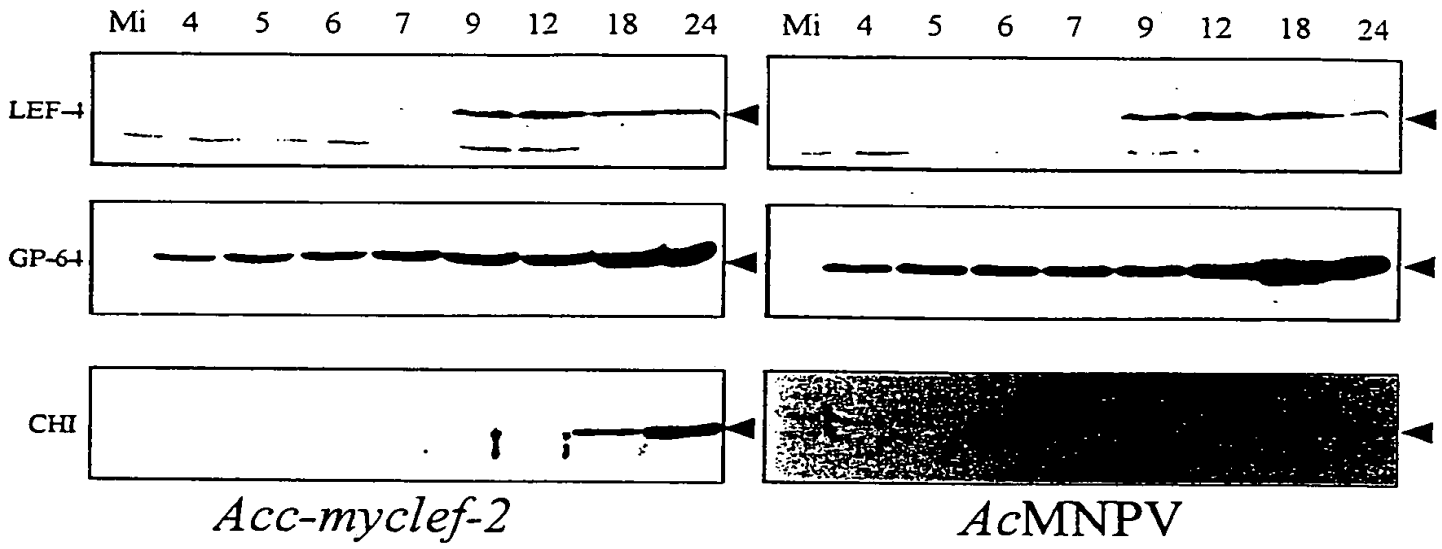
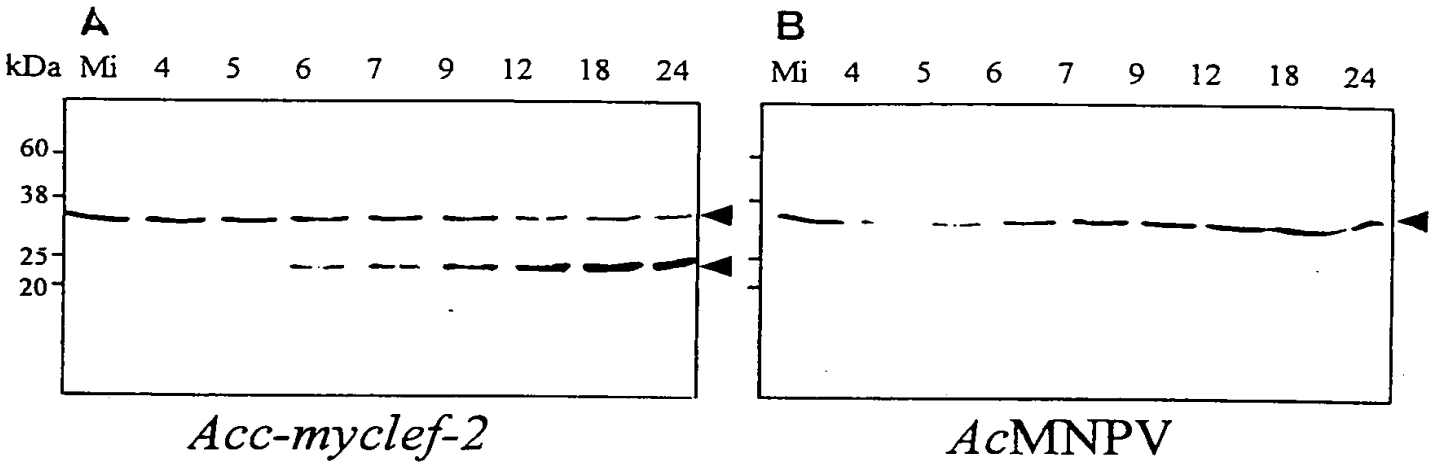

A
A G C T Mi 3 6 12C 12
A A
T G
C G
C C
C G
T T
G T
T
Early promoter
B
A G C T Mi 6 12a 12c 12 24
T C
T T
G T
A A
G T
T A
G T
T
Late promoter

Figure 5



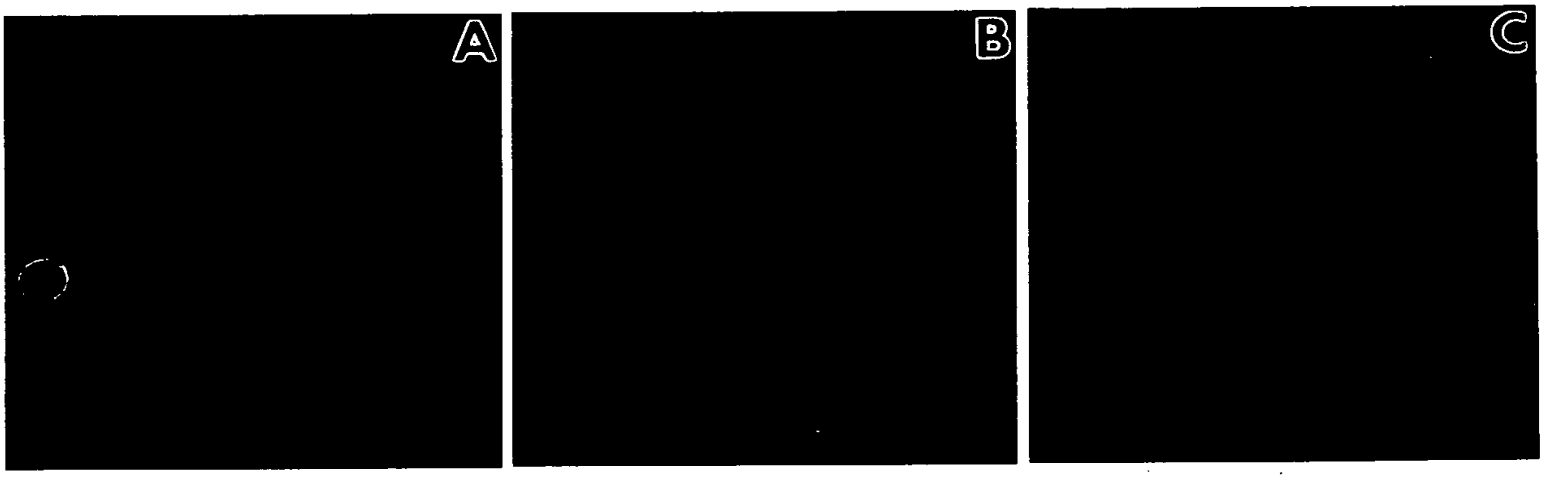
Acc-myclef-2 *Ac*MNPV

Figure 6



Acc-myclef-2 *Ac*MNPV

Figure 7



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